Laccase Down-Regulation Causes Alterations in Phenolic Metabolism and Cell Wall Structure in Poplar[1]

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Laccases are encoded by multigene families in plants. Previously, we reported the cloning and characterization of five divergent laccase genes from poplar (*Populus trichocarpa*) xylem. To investigate the role of individual laccase genes in plant development, and more particularly in lignification, three independent populations of antisense poplar plants, *lac3*AS, *lac90*AS, and *lac110*AS with significantly reduced levels of laccase expression were generated. A repression of laccase gene expression had no effect on overall growth and development. Moreover, neither lignin content nor composition was significantly altered as a result of laccase suppression. However, one of the transgenic populations, *lac3*AS, exhibited a 2- to 3-fold increase in total soluble phenolic content. As indicated by toluidine blue staining, these phenolics preferentially accumulate in xylem ray parenchyma cells. In addition, light and electron microscopic observations of *lac3*AS stems indicated that *lac3* gene suppression led to a dramatic alteration of xylem fiber cell walls. Individual fiber cells were severely deformed, exhibiting modifications in fluorescence emission at the primary wall/middle lamella region and frequent sites of cell wall detachment. Although a direct correlation between laccase gene expression and lignification could not be assigned, we show that the gene product of *lac3* is essential for normal cell wall structure and integrity in xylem fibers. *lac3*AS plants provide a unique opportunity to explore laccase function in plants.

Laccases, or *p*-diphenol:O_2 oxidoreductases (EC 1.10.3.2), are copper-containing glycoproteins found in a wide range of living organisms including bacteria, fungi, insects, and plants. Fungal laccases are by far the most extensively studied group and have been shown to be associated with a large number of physiological processes including morphogenesis, pathogenicity, and lignin degradation (Thurston, 1994). In plants, the role of laccases has never been clearly demonstrated. Several lines of evidence suggest their involvement in lignin biosynthesis. This is based on their capacity to oxidize lignin precursors (*p*-hydroxycinnamyl alcohols), and their localization in lignifying xylem cell walls (Davin et al., 1992; Driouich et al., 1992; Sterjiades et al., 1992; Bao et al., 1993; McDougall and Morrison, 1996). However, without functional proof by genetic modification, this evidence remains circumstantial.

One of the major obstacles encountered in elucidating the function of a given plant laccase is the existence of multiple isoenzymes with overlapping substrate specificities within a given organ or tissue (Mayer, 1987; Sterjiades et al., 1996). Moreover, many other biochemically related phenol oxidases have also been characterized (Chabanet et al., 1994; Liu et al., 1994; McDougall and Morrison, 1996). Among them, coniferyl alcohol oxidases have been the most extensively studied (for review, see Savidge et al., 1998). Coniferyl alcohol oxidases are catechol oxidases that are spatially and temporally associated with active lignification during wood formation in conifers. Based on substrate specificities and protein microsequence data, these enzymes appear to be biochemically and genetically different from laccases (Udagama-Randeniya and Savidge, 1995). However, full-length cDNA sequences are not yet available.

Laccases are encoded by multigene families in Arabidopsis, rice (*Oryza sativa*), *Liriodendron tulipifera* (Lafayette et al., 1999), and *Zinnia elegans* (E. Pesquet, personal communication). Recently, we described the isolation and characterization of five divergent laccase cDNAs (*lac1*, *lac2*, *lac3*, *lac90*, and *lac110*) from *Populus trichocarpa* (Ranocha et al., 1999, 2000). These genes were obtained from a xylem-enriched cDNA library and were most highly expressed in stem tissue. At least two of the five genes, *lac90* and *lac110*, encode proteins that are capable of oxidizing lignin precursors in vitro. Recently, a large scale-sequencing project was initiated using this library as well as a cambial zone cDNA library to obtain genetic information toward understanding wood formation in poplar (*Populus tremula* × *Populus tremuloides* and *P. trichocarpa*; Sterky et al., 1998). Interestingly, of the

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866 xylem expressed sequence tags sequenced from the xylem library, 14 exhibited significant homologies with known laccases. Together, these data suggest that laccases may play a role in some aspect of secondary cell wall formation and/or lignification.

To gain further knowledge as to laccase function in plants, we generated four populations of transgenic poplars, each harboring an individual poplar laccase gene in the antisense (AS) orientation. With the exception of preliminary data of laccase repression in *L. tulipifera* (Dean et al., 1998) and Arabidopsis (Halpin et al., 1999), this is, to our knowledge, the first in-depth characterization of genetic manipulation of laccases in plants. Although none of the four populations showed alterations in lignin content or composition, one of the populations, *lac3*AS, exhibited quantitative differences in global phenolic metabolism accompanied by perturbations in xylem fiber cell wall structure. Although these results do not clearly point to a role of laccases in lignification per se, they do suggest that the product of *lac3* is essential for cell wall structure and integrity in xylem fibers.

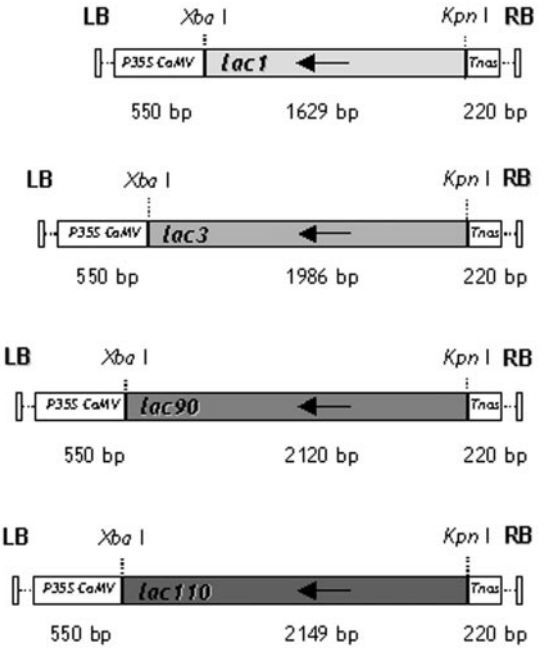

Figure 1. Structures of poplar laccase (*lac1*AS, *lac3*AS, *lac90*AS, and *lac110*AS) AS constructs. P35S CaMV, CaMV 35S RNA promoter; Tnos, termination sequence of the nopaline synthase gene.

RESULTS

Generation and Characterization of AS Laccase Poplar Lines

To gain insight into laccase function in plants, we used an AS RNA strategy to investigate the phenotypic repercussions resulting from suppressing laccase expression. Five different laccase cDNA sequences (*lac1*, *lac2*, *lac3*, *lac90*, and *lac110*) have been previously isolated from a poplar xylem cDNA library (Ranocha et al., 1999). Because *lac2* and *lac3* were extremely homologous (90% at the nucleic acid level), only *lac3* was selected in addition to *lac1*, *lac90*, and *lac110*, for a total of four constructs.

Either full-length (*lac90* and *lac110*) or nearly full-length (*lac1* and *lac3*) poplar cDNA was cloned in AS orientation in the binary vector pJR1 under the control of the 35S cauliflower mosaic virus promoter (CaMV). The resulting constructs were used for *Agrobacterium tumefaciens*-mediated transformation of poplar (Fig. 1). Four AS populations were generated: *lac1*AS, *lac3*AS, *lac90*AS, and *lac110*AS. The integration of an AS laccase transgene in primary transformants was determined by PCR on genomic DNA (data not shown). Transformed, in vitro-micropropagated plantlets (10–15 independent transformants per population) were then acclimated in growth chambers for 2 months for subsequent analysis. Screening, performed by northern-blot analysis, indicated that of the four constructs, only three gave rise to significant laccase gene suppression. We were unable to generate *lac1*AS poplars with a significant reduction in *lac1* gene expression.

For all three populations, *lac3*AS, *lac90*AS, and *lac110*AS, transformants exhibited a varying degree of down-regulation as compared with controls. Results of northern-blot screening of *lac3*AS, *lac90*AS, and *lac110*AS poplars are illustrated in Figure 2. For *lac3*AS poplars (Fig. 2A), the larger transcript of approximately 2.2 kb corresponds to the endogenous *lac3* transcript and is present in similar amounts in both untransformed (C1) and transformed (C2) controls. The most significant reduction was observed in *lac3.2*AS and *lac3.4*AS, exhibiting barely detectable residual levels of *lac3* endogenous transcript.

Because a truncated cDNA was used for *lac3*AS transformation, the AS transcript on northern blots migrated as a smaller (2.0 kb), distinct band as compared with its endogenous counterpart. In a few transformants (*lac3.3*AS and *lac3.4*AS), the *lac3* AS transcript was highly expressed. However, no correlation was observed between the level of transgene expression and extent of laccase gene suppression. For example, *lac3.2*AS is characterized by a nearly undetectable level of endogenous *lac3* messenger with a low AS transcript level, whereas in the *lac3.3*AS line, the AS transgene is highly expressed without a significant reduction in endogenous *lac3* expression (Fig. 2A).

For *lac90*AS and *lac110*AS poplars (Fig. 2, B and C, respectively) the AS transcript and the endogenous messenger are the same size. Therefore, the double-stranded DNA probe used to screen these plants did not differentiate sense from AS transcript. A unique signal of 2.2 kb resulting from these two types of transcripts was detected by northern blot. *Lac90*AS and *lac110*AS plants with the lowest "sense + AS" to rRNA ratio (i.e. *lac90.4*AS, *lac90.13*AS, and *lac110.6*AS)

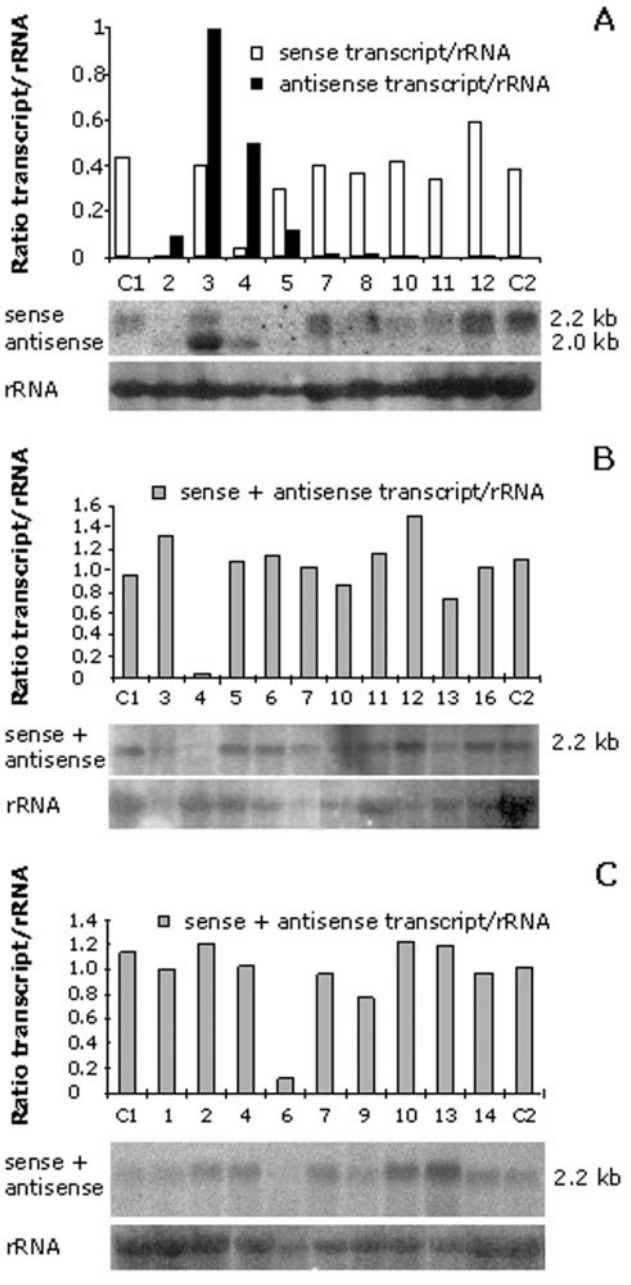

Figure 2. Molecular screening of laccase AS poplar transformants by northern-blot analysis. Twenty-five micrograms of total RNA from *lac3*AS, *lac90*AS, and *lac110*AS stems (A, B, and C, respectively) were electrophoresed on denaturing gels and probed with the corresponding radiolabeled *lac3*, *lac90*, or *lac110* 3′-untranslated region (UTR). In A, histograms represent levels of endogenous (white bars) and AS transgene (black bars). In B and C, gray bars represent endogenous + transgene expression. In all cases, signals were normalized with respect to 28S ribosomal RNA content. Controls, In vitro-propagated untransformed poplars (C1) and poplars transformed with empty pJR1 vector (C2).

along with *lac3.2*AS, *lac3.3*AS, and *lac3.4*AS, were selected for further analysis. None of these selected transformants exhibited any readily visible phenotypic variation (i.e. plant height, stem thickness, and phyllotaxy) as compared with controls.

Down-Regulation of Laccases Does Not Modify Lignin Content or Structure

Lignin analysis was carried out on cell wall residues (CWR). Because there is no given method that can be regarded as totally satisfactory for the quantification of lignin, two methods were used to evaluate lignin content of transgenic and control lines: the spectroscopic acetyl bromide method (Iiyama and Wallis, 1988) and the gravimetric micro-Klason procedure (Whitting et al., 1981). As determined by both methods, none of the AS laccase transgenics exhibited a significant decrease in lignin content as compared with control poplars (Table I). Lignin content was 20% to 25% of the CWR on a dry weight basis. These values are in agreement with those previously reported in poplar (Baucher et al., 1996).

Lignin composition was then investigated by gas chromatography analysis of thioacidolysis products. This method selectively cleaves labile ether bonds interconnecting lignin monomeric units (namely β-*O*-4 linkages) and therefore enables the determination of monomeric composition of non-condensed lignin. The total yield of monomers (syringyl + guaiacyl) recovered by thioacidolysis was not significantly different in AS poplars as compared with controls (1,291 $\pm$ 166 μmol g^{-1} lignin versus 1,330 $\pm$ 94 μmol g^{-1} lignin, respectively). Moreover, the syringyl to guaiacyl ratio was not significantly altered by laccase down-regulation (1.99 $\pm$ 0.08 for control plants and 2.02 $\pm$ 0.06 for AS plants).

AS Suppression of *lac3* Leads to an Increase in Ethanol-Soluble Phenolic Content

Ethanol-soluble phenolic compounds were quantified based on their reactivity vis-à-vis Folin's reagent. Both *lac3.2*AS and *lac3.4*AS exhibited a 2- to 3-fold increase in soluble phenolic content (Fig. 3). No in-

Table I. *Lignin content of laccase antisense poplars*

Individual[a]	Micro-Klason[b]	Acetyl Bromide
	% CWR	
C1	20.7 $\pm$ 0.6	23.9 $\pm$ 1.1
C2	20.8 $\pm$ 0.7	24.3 $\pm$ 1.5
*lac3.2*AS	20.9 $\pm$ 0.5	25.5 $\pm$ 0.9
*lac3.3*AS	20.5 $\pm$ 0.6	23.6 $\pm$ 1.1
*lac3.4*AS	20.1 $\pm$ 0.7	23.4 $\pm$ 0.6
*lac90.4*AS	19.9 $\pm$ 0.6	25.3 $\pm$ 0.4
*lac90.13*AS	20.5 $\pm$ 0.7	25.3 $\pm$ 0.8
*lac110.4*AS	21.4 $\pm$ 0.8	25.4 $\pm$ 0.7
*lac110.6*AS	19.3 $\pm$ 0.4	25.2 $\pm$ 0.4

[a] C1, Untransformed poplars, C2, poplars transformed with empty vector. [b] Values are given as a percentage of the CWR (see "Materials and Methods") and correspond to the mean of four determinations ($\pm$SD).

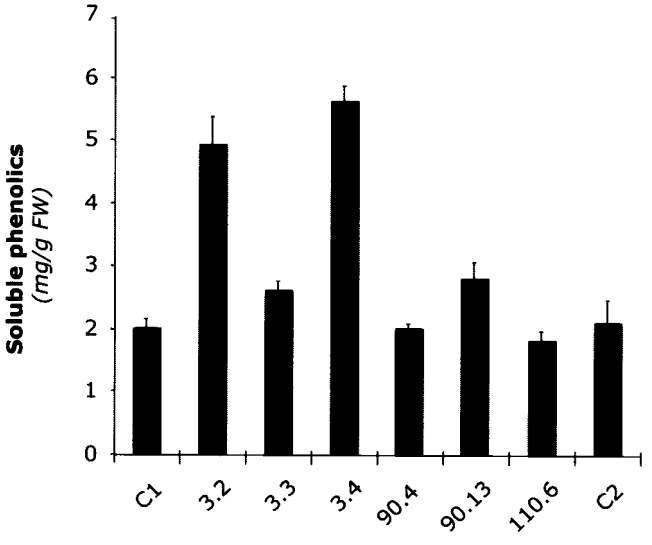

Figure 3. Soluble phenolic content in AS laccase and control poplar plants. Data are presented as means (±SD) of three separate measurements. In abscissa, numbers (3, 90, and 110) before the dot refer to the name of the laccase clone introduced in AS orientation. As in Figure 2, C1 and C2 are controls (untransformed poplars and poplars transformed with empty pJR1 vector, respectively).

crease was observed for *lac3.3*AS poplars. Interestingly, this line was selected for its high level of transgene expression; its endogenous *lac3* expression was comparable with control levels (see Fig. 2). *lac90*AS and *lac110*AS poplars did not exhibit significantly different soluble phenolic content as compared with controls.

To determine if the observed increase in soluble phenolics in *lac3*AS plants was due to a uniform increase in all phenolics or uniquely due to variations in a particular metabolite or subset, soluble phenolics of *lac3.2*AS, *lac3.4*AS, and control plants were separated by HPLC. From a qualitative standpoint, *lac3.2*AS and *lac3.4*AS profiles were nearly identical to controls with the exception of one peak (indicated by an arrow on Fig. 4) that was present in AS plants but absent in controls. Unfortunately, we were not able to identify the corresponding compound. In both AS lines, a preferential accumulation of certain phenolics (i.e. peaks 2, 3, and 5) was observed (Fig. 4). The major peaks were further analyzed by MS. Peaks 2, 3, and 5 were identified as salicortin, salireposide, and tremulacin, respectively (Fig. 4).

In addition, alkali-soluble cell wall phenolics were analyzed. A number of components were present in the samples and about one-half of them (i.e. *p*-OH-benzoic acid, *p*-OH-benzaldehyde, vanillic acid, vanillin, *p*-coumaric acid, ferulic acid, cis ferulic acid, and 8-O-4 diferulic acid) were identified on the basis of their retention time and spectral properties. No significant changes with respect to these phenolics were detectable in *lac3.*AS plants as compared with control poplars (data not shown).

lac3 Is Necessary for Normal Cell Wall Structure and Integrity in Xylem Fibers

Microscopic observations of *lac3.2*AS and *lac3.4*AS revealed that the overall pattern of xylem tissue as seen in transverse sections was not dramatically disorganized as compared with control sections (Fig. 5). All of the different cell types were easily recognizable: xylem vessels, fibers, and ray parenchyma cells. Measurements of mean cell area of xylem fibers indicated that there was no significant difference in cell size between *lac3*AS (*lac3.2*AS = 110.3 ± 2.3 μm^2 and *lac3.4*AS = 107.3 ± 1.6 μm^2, n = 450) and control (112.9 ± 3.4 μm^2, n = 186) poplars. The walls of xylem vessels and fibers from both control and AS plants were lignified, as indicated by a characteristic blue fluorescence under UV excitation and phloroglucinol staining. However, it was readily observed that the walls of *lac3.2*AS and *lac3.4*AS xylem fibers possessed a highly irregular cell contour as compared with controls (Fig. 5, A–C). Similar irregularities in cell shape were also observed in the xylem of *lac3*AS roots (data not shown). Moreover, in AS plants, the fluorescence emission was not homogeneous throughout the entire width of the wall, and fluorescence was negligible in the middle lamella/primary wall region between adjacent fibers. As a consequence, the cells appeared to be detached from one another. The intensity of phloroglucinol staining was not altered in *lac3*AS plants (Fig. 5, D–F). This is in agreement with the fact that no quantitative or qualitative differences in lignin were detected in these plants.

To further characterize *lac3*AS cell wall structure, detailed light and electron microscopic observations were performed (Fig. 6). When cross sections were treated with toluidine blue, *lac3*AS lines exhibited an increase in the intensity of dark blue staining in xylem ray parenchyma cells as compared with control plants (Fig. 6, A and B). This coloration indicates an accumulation of phenolic compounds in these cells (Ros Barcelo et al., 1989). These results not only reinforce the quantitative data of soluble phenolics above but they also point to cell-specific phenolic accumulation. At the electron microscope level, defects in cell wall structure of xylem fibers were clearly visible. In controls (Fig. 6C), as expected, the secondary walls were firmly laid down on the primary walls of xylem fiber cells. In contrast, in *lac3*AS plants, adhesion defects occurred either at the primary cell wall of adjacent cells or within the secondary wall of a given cell (Fig. 6, D–F).

An increase in cell wall fragility in *lac3* downregulated plants suggested that the mechanical properties of stems had also been altered. Toward this end, microtome-cutting tests were performed on the second to third internodes of *lac3.2*AS and *lac3.4*AS plants. Control plants exhibit a greater cutting work than *lac3.2*AS and *lac3.4*AS plants to fracture the wood: 207 ± 51 J m^{-2} for control plants versus 97 ±

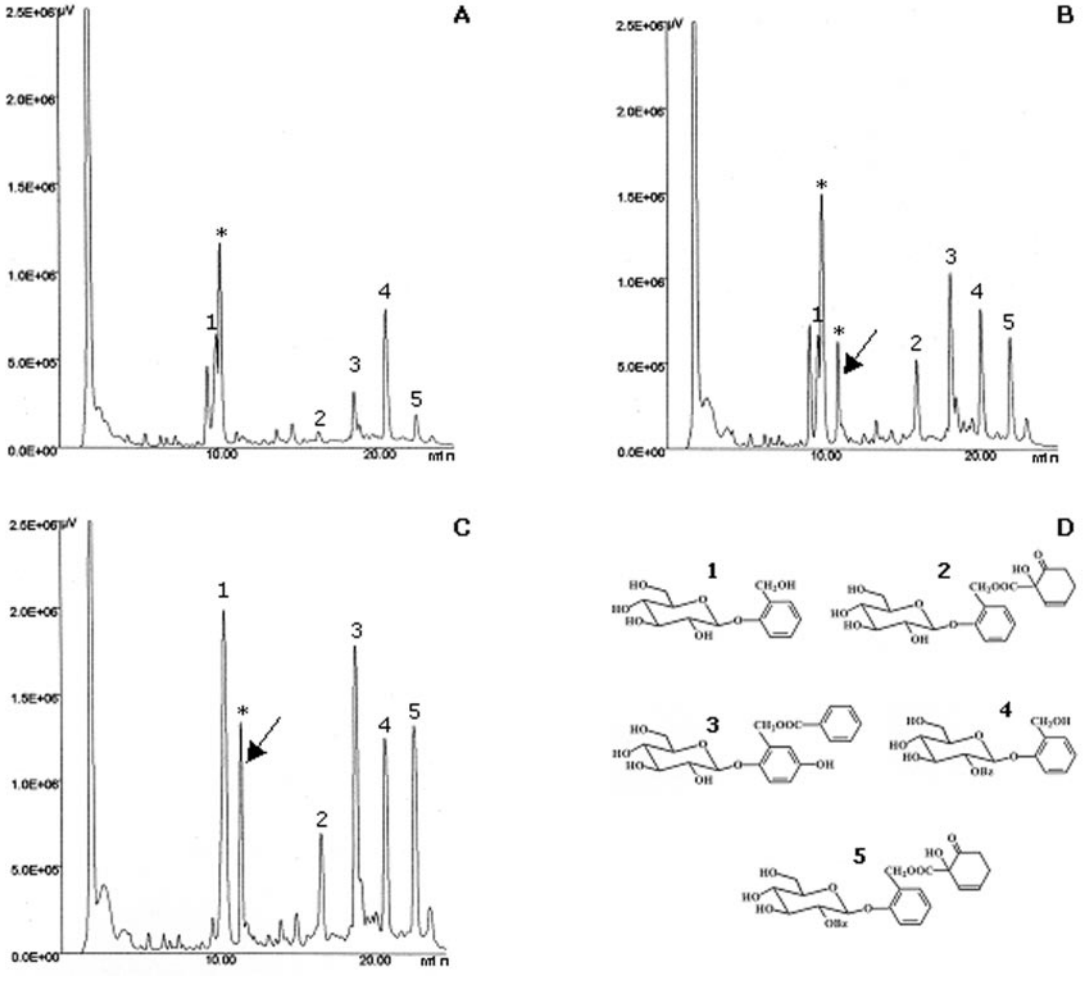

Figure 4. HPLC profiles of total soluble phenolics of control (A), *lac3.2*AS (B), and *lac3.4*AS (C) plants. The numbered peaks were identified by HPLC-mass spectrometry (MS). The corresponding chemical structures of the peaks identified by HPLC-MS are indicated in D and are: salicin (1), salicortin (2), salireposide (3), tremuloidin (4), and tremulacin (5; Clausen et al., 1989). Peaks marked with an asterisk could not be identified by MS. An arrow indicates a peak that was present in AS lines but not in controls. Bz, Benzoate.

23 J m^{-2} for *lac3.2*AS and 115 ± 42 J m^{-2} for *lac3.4*AS plants. These results indicate that the modification of *lac3* gene expression leads to significant alterations of the mechanical properties of wood.

DISCUSSION

Although the exact mechanisms of the AS RNA technology have not yet been fully elucidated, this approach has been used with success to reduce the expression of a number of plant genes (Watson and Grierson, 1993), including genes involved in lignin biosynthesis (for review, see Campbell and Sederoff, 1996; Grima-Pettenati and Goffner, 1999). We report here the first in-depth analysis of plants with genetically reduced levels of laccase expression. This has been achieved by expressing individual members of the laccase gene family in AS orientation in poplar. Preliminary data have been reported on laccase down-regulation in *L. tulipifera* (Dean et al., 1998), and Arabidopsis (Halpin et al., 1999) but to our knowledge, they have not been further substantiated.

Plants were screened by northern-blot analysis because it was not possible to measure laccase activity in crude extracts of poplar xylem/stem tissue. Detection of laccase activity in crude plant extracts is a problem that has often been encountered (Savidge and Udagama-Randeniya, 1992; McDougall and Morrison, 1996). Crude extracts may contain either a natural oxidase inhibitor or an antioxidant that may interfere with activity measurements. In this study, three populations of transformants with severely down-regulated levels of laccase expression, *lac3*AS, *lac90*AS, and *lac110*AS, were successfully obtained.

Our main objective was to determine the consequences of laccase down-regulation on lignin content and composition. It has been previously demonstrated that laccases from several plant species efficiently oxidize monolignol precursors of lignin in vitro and are temporally and spatially correlated

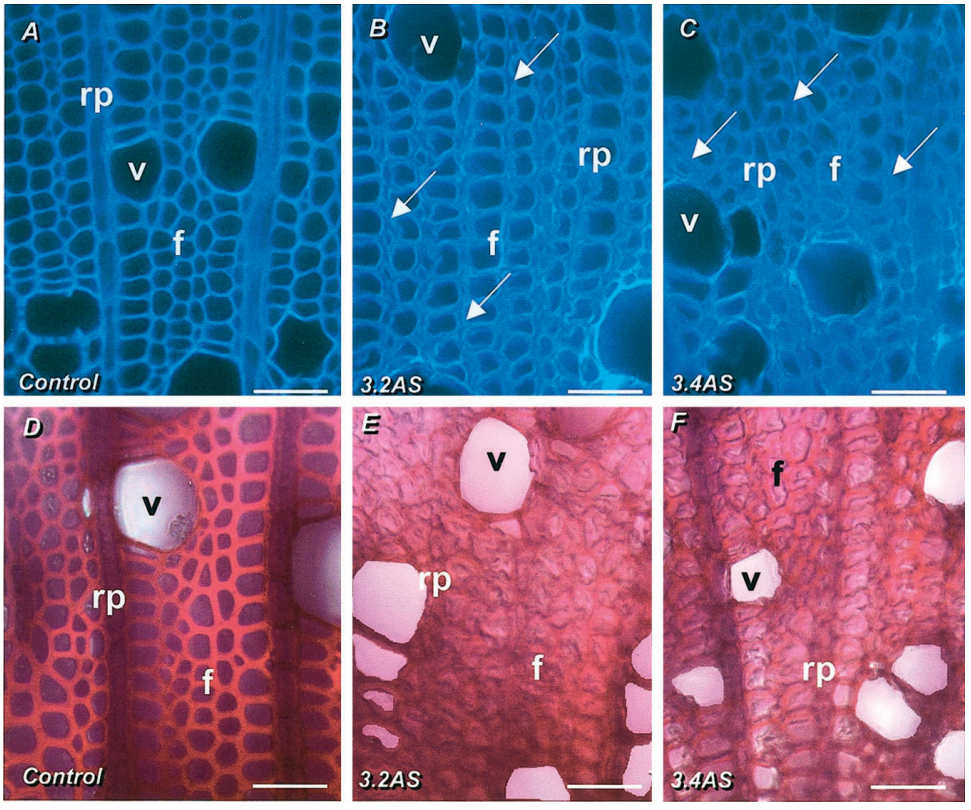

Figure 5. Transverse stems sections of poplar visualized by UV fluorescence microscopy (A–C) and phloroglucinol staining (D–F). A and D, Control (transformed with empty vector); B and E, *lac3.2*AS; C and F, *lac3.4*AS. v, Xylem vessel; rp, ray parenchyma; f, xylem fiber. Note the deformed cell contour of xylem fibers and an absence of fluorescence emission (arrows) at the middle lamella/primary wall region in *lac 3.2*AS (B) and *lac3.4*AS (C) as compared with control fibers (A). Scale bars = 50 μm.

with lignin deposition (Driouich et al., 1992; Sterjiades et al., 1992; Bao et al., 1993). Although these criteria make laccases viable candidates for involvement in lignin biosynthesis, we sought to provide functional evidence, via genetic modulation of laccase expression, to support or refute the hypothesis of laccase participation in lignin biosynthesis. Despite a severe reduction in levels of individual laccase gene expression, none of the transformants exhibited significant differences in either lignin content or composition. There are several possible explanations for these results. First, there may be sufficient residual laccase activity to allow for normal lignification. It is possible that under normal conditions, plants produce laccase in far greater amounts than required for constitutive lignification. This explanation is always a possibility in AS experiments that do not result in the predicted outcome because AS experiments never results in 100% gene extinction. Recently, Arabidopsis laccase mutants have been identified in a T-DNA insertional mutant collection by systematic sequencing plant DNA adjacent to the insertion (Tissier et al., 1999). The characterization of these putative knockouts may provide an invaluable tool to elucidate the role of these particular laccases in Arabidopsis.

Another likely explanation may be that the silencing effect of a given laccase gene is masked by the functional compensation of a divergent laccase and/or broad-spectrum oxidase that is not affected by the AS transgene. This would require that the enzyme(s) that "takes over" either shares the same constitutive spatio-temporal expression as the repressed gene or can be induced in the affected cell types in down-regulated plants. These difficulties are commonly encountered when elucidating the role of individual members of multigene families. One noteworthy example was provided in tobacco (*Nicotiana tabacum*) transgenics underexpressing an anionic peroxidase gene (Lagrimini et al., 1997). It had been shown previously that the overexpression of this anionic peroxidase led to an increase in lignin content (Lagrimini, 1991), thereby suggesting a role in lignification. Therefore, it was unexpected that tobacco plants with severely reduced levels of this peroxidase did not lead to alteration in lignin content or quality (Lagrimini et al., 1997). Interestingly, genomic and expressed sequence tag sequencing projects have revealed that a relatively large proportion of known cell wall-related proteins, including cellulose synthases, xyloglucan endo-transglycosylases, and ex-

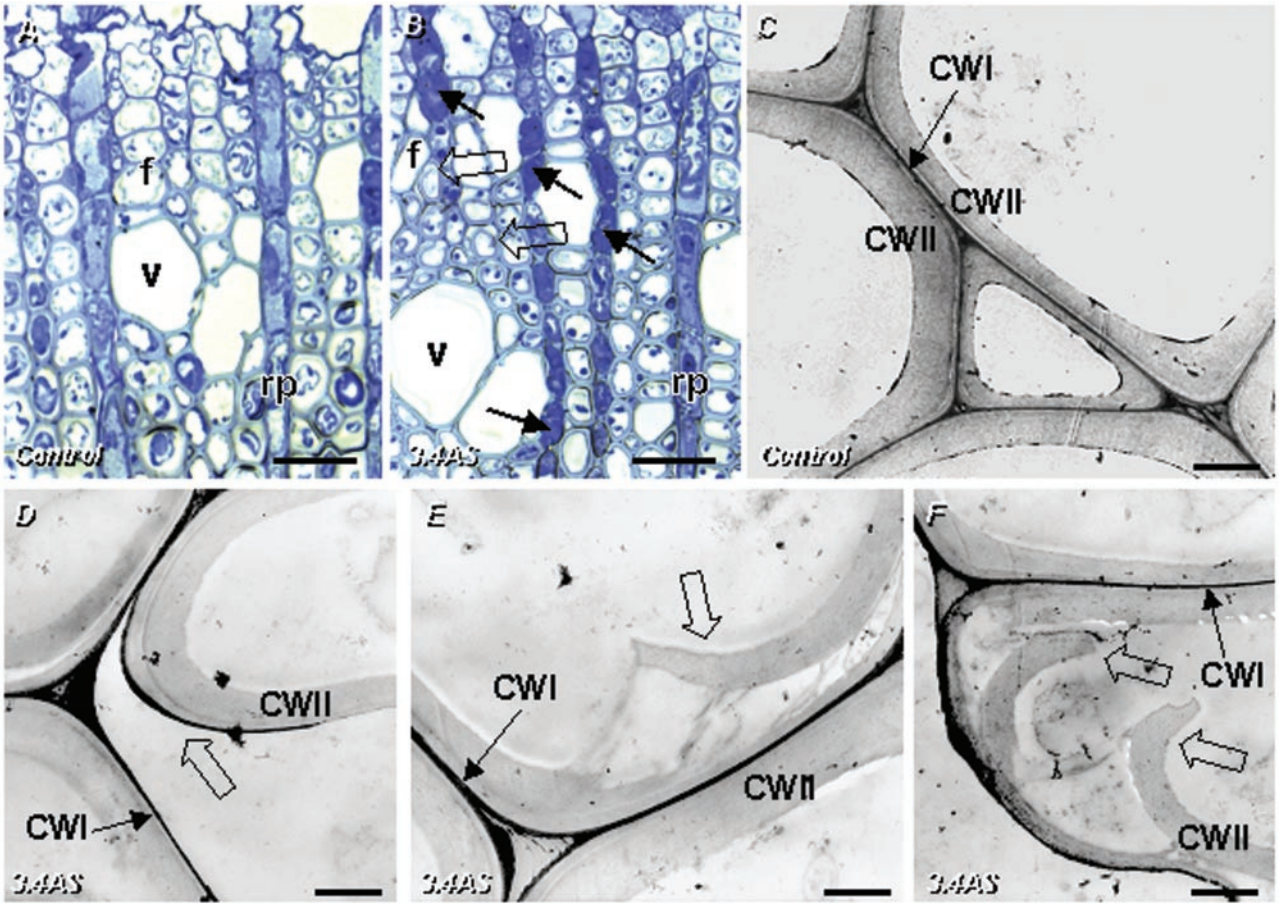

Figure 6. A and B, Transverse sections of poplar stained with toluidine blue. A, Control; B, *lac3*.4AS. Note the accumulation of dark-blue stain in xylem ray parenchyma cells and the detachment of cell walls (indicated by black and white arrows, respectively). Scale bars = 50 μm. Abbreviations are as in Figure 5. C through F, Electron micrographs of xylem fibers in control (C) and *lac3.4*AS (D–F) plants. Note detachment of primary (D) and secondary (E and F) cell walls (indicated by open arrows). CWI, Primary wall; CWII, secondary wall. Scale bars = 2 μm in C and 1 μm in D through F.

pansins, are encoded by large multigene families (Fagard et al., 2000). This provides an additional challenge for researchers in unraveling the mechanisms controlling cell wall structure and function. Toward this end, the functions of three cDNAs encoding cellulose synthase genes have recently been explored using virus-induced gene silencing in *Nicotiana* spp. (Burton et al., 2000).

One may not eliminate the hypothesis that the participation of laccases in lignification is too subtle to be detected by global analyses used to measure lignification in whole stem tissue. It has now become widely accepted that lignin content and composition is dictated by a complex array of cell-specific regulatory mechanisms. Several lignification enzymes encoded by multigene families, including 4-coumarate: coenzyme A ligases (Hu et al., 1998) and Caffeoyl coenzyme A *O*-methyltransferase (Chen et al., 2000) exhibit extremely precise cell-specific expression patterns. In this context, it is possible that a given laccase is important for lignin deposition exclusively in certain specialized cell types. Hoopes et al. (1995) demonstrated a spatio-temporal regulation of high- and low-pI laccases in *Z. elegans* stems. A high-pI isoform was expressed in xylem vessels, whereas low-pI laccases were associated with libriform (xylem) and sclerenchymal (phloem) fibers. In the future, a more in-depth knowledge of cell-/tissue-specific expression of laccases, together with micro-, in situ techniques for lignin analysis will be crucial in understanding the veritable role of laccases in wall formation. It is also possible that certain laccases are associated with lignin deposition only under certain precise physiological conditions. Although we have previously shown that *lac110* is constitutively expressed in poplar xylem (Ranocha et al., 1999), Pilate et al. (2000) have demonstrated using cDNA-amplified fragment-length polymorphism that this laccase is significantly up-regulated in tension wood, thereby suggesting a potential role for laccase in the formation of "stress" lignin in poplar.

Another conclusion that cannot be ruled out from the results presented here is that laccases, or at least the ones examined herein, are not involved in ligni-

fication. This viewpoint was supported by Ros Barcelo (1995), who postulated that peroxidase was the sole enzyme involved in the ultimate step of lignin biosynthesis, but most recent reports on lignin biosynthesis are more moderate and envisage the involvement of both oxidases in the formation of phenoxy radicals.

Based on the results obtained with *lac3*AS poplars, we postulate that one of the many putative roles of laccases in plants may be to assure proper cell wall structure in xylem fibers. Based on the high degree of homology between *lac2* and *lac3*, it could have been possible that *lac2* expression was also repressed in *lac3*AS plants. The simultaneous repression of two genes would have explained, in part, why we were able to observe a phenotype in *lac3*AS plants. However, this was not the case because *lac2* expression in *lac3*AS plants was equivalent to wild-type levels, as assessed by northern-blot hybridization (data not shown). The fact that an accumulation of soluble phenolic compounds was observed in *lac3*AS poplars indirectly implies that some phenolic compounds were not properly polymerized at the cell wall. This is supported by the finding that there was a decrease in fluorescence in the primary wall/middle lamella region of neighboring xylem fibers. This resulted in a detached appearance of xylem fibers. Interestingly, an analogy may be made with a fungal laccase from *Armillaria* spp. that is responsible for accumulation of melanin-like polyphenols in the intercellular spaces during rhizomorph formation (Worral et al., 1986). This laccase-mediated accumulation of extracellular phenolic material was correlated with a decrease in amount of soluble phenolics. The authors concluded that the strengthening of cell-cell adhesion via intercellular polymerization of polyphenols might well be one of the essential functions of laccases in fungi.

In plants, it is now well established, especially in grasses, that cell wall phenolics play a critical role in cross-linking wall components (for review, see Iiyama et al., 1994; Carpita, 1996; Ralph et al., 1998b). In non-lignified cell walls, cross-linking may occur as a result of dehydrodiferulic acid formation between neighboring ferulic acid residues esterified on wall polysaccharides (Ishii, 1991). In lignified cells, hydroxycinnamic acid-mediated cross-linking also occurs between polysaccharides and lignins (Ralph et al., 1995). Polysaccharide-ferulate esters in the cell wall undergo radical coupling reactions with neighboring ferulate residues to produce a wide range of diferulates. These coupling reactions likely involve wall-associated oxidases such as peroxidases and/or laccases. For example, diethyl 8-5 diferulate may be synthesized in vitro by addition of peroxidase and hydrogen peroxide (Ralph et al., 1998a). Thus, it is tempting to speculate that *lac3* down-regulation reduces the formation of certain types of phenoxy radicals and results in a decrease in cross-linking of xylem fibers. The accumulation of soluble phenolic metabolites observed in *lac3*AS poplars is coherent with this hypothesis. Interestingly, dehydrodiferulic acid cross-links have been shown to be implicated in cell aggregation in rice suspension cultures (Kato et al., 1994). Beyond elucidating the role of laccases in plant development, these findings may have important repercussions for the use of laccases in modifying cell wall structure for biotechnological applications related to fiber use.

MATERIALS AND METHODS

Construction of AS Vectors and Poplar (*Populus tremula* × *Populus alba*) Transformation

lac1 (1.6 kb), *lac3* (2.0 kb), *lac90*, and *lac110* (2.1 kb full length) cDNAs were inserted (*Kpn*I-*Xba*I) in the AS orientation into the binary vector, pJR1, under the control of the 35S CaMV promoter. The resulting plasmids were introduced into *Agrobacterium tumefaciens* C58/pMP90 strain according to the method described by Holsters (1978). Poplar transformation (Institut National de la Recherche Agronomique clone 717-1B4), was carried out according to Leplé et al. (1992). Four populations of AS laccase transgenics were produced: *lac1*AS, *lac3*AS, *lac90*AS, and *lac110*AS. As a control, poplars were also transformed with an empty pJR1 vector (plants C2). Transformed poplars were selected for kanamycin resistance. Integration of the AS transgene was confirmed by PCR on genomic DNA with a primer located in the 35S CaMV promoter and gene-specific primer for each gene. In brief, each reaction contained 100 ng of genomic DNA and 10 pmol of each primer. After initial denaturation (94°C, 5 min), PCR was carried out for 30 cycles (94°C, 1 min; 65°C, 1.5 min; and 72°C, 2 min) and terminated by 10 min at 72°C.

AS and controls (both untransformed and poplars harboring an empty pJR1 vector) were micropropagated in vitro (Leplé et al., 1992).

Molecular Screening of AS Laccase Poplar Transformants

In vitro-micropropagated plantlets were grown for 2 months in sterile tubes. They were then cultivated on vermiculite and grown in a culture room (25°C, 16-h-light/8-h-dark photoperiod, 80% relative humidity, light intensity: 80 μE m^{-2} s^{-1}) for 2 months before screening. For northern blots, 25 μg of total RNA from stems (second to fourth internode) of each independent transformant (15 individuals/population) were denatured with glyoxal/dimethylsulfoxide, electrophoresed on 1% (w/v) agarose gels, and blotted onto positively charged nylon membranes (Hybond N^+, Amersham, Buckinghamshire, UK). RNA from stem tissue was used for determining gene expression because it has been previously shown that all of the above laccase genes are most abundantly expressed in this tissue (Ranocha et al., 1999). To probe gene-specific expression, divergent 3′-UTRs of *lac1*, *lac3*, *lac90*, and *lac110* were radiolabeled with (α^{32}P)-dCTP with a Nonaprimer Kit II (Appligene Oncor, Heidelberg) according to the manufac-

turer's recommendations. Probe specificity had been previously verified by the absence of cross hybridization among 3′-UTRs on Southern blots (Ranocha et al., 1999). Membranes were prehybridized in 3× SSC, 0.5% (w/v) SDS, and 0.5% (w/v) low-fat milk for 2 h at 65°C and hybridized overnight under the same conditions. Membranes were then washed with 3× SSC and 0.5% (w/v) SDS for 15 min at 65°C. The resulting hybridization signals were quantified using ImageQuant (PhosphorImager; Molecular Dynamics, Sunnyvale, CA) and normalized with respect to the corresponding amounts of 28S rRNA. Poplars plantlets with reduced levels of laccase gene expression were selected for subsequent characterization.

Lignin Analysis

Stems (second to fourth internode) harvested from 2-month-old control and transgenic lines were weighed and lyophilized. The bark was removed and the remaining material was ball milled for 10 min to a fine powder (<180 μm). The powder was then subjected to successive extractions with water, ethanol, toluene:ethanol (1:1 [v/v]), and acetone using a modified Soxhlet apparatus. The resulting CWR was used for lignin determinations.

Lignin content was determined by two independent methods. Acetyl bromide lignin was determined by the method of Iiyama and Wallis (1988). Klason lignin was determined according to Whitting et al. (1981). For the determination of lignin monomer composition, thioacidolysis was performed as described by Lapierre et al. (1986).

Extraction and Analysis of Total Soluble Phenolic Compounds

Fresh stem tissue (0.5 g) was ground to a fine powder. The powder was then homogenized three times at 4°C in 80% (v/v) ethanol (50 mL). The crude extract was filtered and evaporated at 35°C under reduced pressure. The aqueous fraction was extracted twice with petroleum ether (boiling point 40°C–60°C) to remove pigments, freeze dried, and stored at −20°C until further use.

Total phenolic compounds were determined by the Folin-Ciocalteu method as described by Scalbert et al. (1989). In parallel experiments, 12% (w/v) polyvinylpolypyrrolidone (PVPP) was added to the extract to bind polyphenols. Total soluble phenolics were calculated by subtracting the value obtained for residual, non-phenolic compounds (after treatment with PVPP) from the total value obtained without addition of PVPP.

Total soluble phenolic compounds were separated by HPLC. Extracts were lyophilized and then dissolved in 0.5 mL of water and filtered before injection onto a C18-silica column (Waters Nova-Pak, 4 μm, 25 cm in length, Spectra-Physics, Mountain View, CA). The flow rate was 1 mL min^{-1} and the column was maintained at 35°C. Phenolic compounds were eluted using a linear gradient of water-methanol (gradient 0%–100% [v/v]) at room temperature and detected at 220 nm.

Identification of Phenolic Compounds by HPLC-MS

Compounds were first identified by comparing HPLC elution times of coinjected samples and standards (Picard, 1994). To confirm data, the extracts were analyzed by HPLC-electrospray ionization MS (Finnigan LCQ mass spectrometer). The heated capillary and voltage were maintained at 200°C and 4.5 kV, respectively. Tandem MS was performed using helium as the collision gas.

Microscopic Techniques

Hand sections from fresh poplar stems (fourth internode) were made with a razor blade and observed using an inverted microscope (Leitz DMIRBE, Leica, Rueil-Malmaison, France) equipped with epifluorescence illumination (Excitation filter BP 340–380 nm, suppression filter *LPI* 430 nm). Images were registered using a CCD camera (Color Coolview, Photonic Science, East Sussex, UK) and treated by image analysis (Image Pro-Plus, Media Cybernetics, Silver Spring, MD).

For the preparation of semithin (2 μm thick) and ultrathin sections (70–80 nm thick), samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 h at room temperature. They were then dehydrated in a graded aqueous ethanol series (20%, 40%, 60%, 80%, and 100% [v/v]; each step was performed twice for 15 min each). Sections were then gradually infiltrated with LR White resin (2:1, 1:1, and 1:2) with ethanol (v/v), and then in 100% LR White overnight. They were then embedded in gelatin capsules and polymerized for 24 h at 60°C. Semithin sections were mounted on glass slides and stained with toluidine blue O (0.5% [w/v] toluidine blue O CI 52040 in 2.5% [w/v] Na-carbonate, pH 11). Polyphenolic compounds were stained dark blue (Ros Barcelo et al., 1989). For transmission electron microscopy, ultrathin sections were cut with a diamond knife using an UltracutE microtome (Leica) and collected on gold grids. Sections were labeled with PATAg to visualize polysaccharides (Thiery, 1967) and observed at 80 kV with an electron microscope (H600, Hitachi, Tokyo).

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